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The factors governing sexual reproduction in the Mucorineae have been regarded by various writers as due to the nutritive characters of the medium, the humidity of the surrounding atmosphere, the oxygen supply, and, lastly, the presence of conjugating male and female strains. So conflicting have been the theories that it is evident that the conditions controlling the production of zygospores are not so simple as many persons have supposed.

When EHRENBERG first discovered the zygospores of *Sporodinia grandis*, he regarded their formation as a process comparable to conjugation in *Spirogyra*. Since it was noticed that among the Mucorales zygosporic formation did not always occur, different workers gave their attention to the factors controlling their production. DEBARY, after working with *Rhizopus*, came to the conclusion that the lack of oxygen was the controlling factor. He found that the zygospores were produced in a closed tube more abundantly than in a tube opened to the air. VAN TIEGHEM repeated the work of DEBARY, and confirmed his views that desiccation could account for zygosporic formation in *Absidia septata*. On the other hand, he believed the zygospores described by BREFELD, in *Piptocephalis* and *Sporodinia*, were brought about by unfavorable food supply. BAINIER also thought that the environment influenced sexual reproduction, but he maintained that the formation of sexual organs was dependent on a nutritious rather than a poor substratum. ZOPF found *Pilobolus* producing zygospores, but ascribed their production to the fact that it was attacked by the parasites *Pleotrachelus fulgens* and *Syncephalis* sp. KLEBS maintained that their formation is induced by increased humidity, which hinders transpiration. FALCK, on the other hand, found that humidity and transpiration within normal limits have no effect on the production of zygospores. BREFELD maintained an agnostic attitude, and in a series of papers denied that

[1] Contribution from the Cryptogamic Laboratories of Harvard University, no. 84.

the environments were, in themselves, sufficient to induce zygospore formation.

The early mycologists believed that all the genera were alike in their method of zygospore formation, but that their irregularity of occurrence was to be accounted for by one factor or a combination of the factors mentioned. BLAKESLEE, however, in 1904, showed that the Mucorineae could be divided into 2 classes, namely, homothallic and heterothallic forms, the homothallic forms being those whose zygospores are formed by the conjugation of gametes which are produced by hyphae of the same individual mycelium. To this group belong such genera as *Dicranophora*, *Sporodinia*, *Spinellus*, *Zygorynchus*, and some species of the genus *Mucor*. The heterothallic forms are those in which 2 kinds of individual mycelia occur. If the 2 individual strains of a heterothallic form are grown on the same medium, zygospores are produced at the point where the hyphae of the 2 strains meet. In this group belong most of the genera of the Mucorineae. Those 2 strains BLAKESLEE first called plus and minus, but in later papers he states that the sexes are as distinct as in higher organisms; the plus he calls female and the minus male. In the *Biological Bulletin* for August 1915 (p. 87) he says: "Conjugation in the Mucors is as definitely a sexual process as the morphologically more complex types of reproduction in higher forms, and the sexes seem even more sharply distinct." In the homothallic form *Zygorynchus heterogamus* one gamete is larger than the other; BLAKESLEE calls the larger gamete female and the smaller male. If the gametes are strictly male or female, as he maintains, there must have been a segregation of the male and the female nuclei in the respective gametes. In the heterothallic forms the male and female nuclei are segregated in different mycelia, and all the gametes produced by a single mycelium, therefore, must be either male or female.

According to BLAKESLEE, whenever a plus strain meets a minus strain of the same species, zygospores are produced. When opposite strains of different species meet, progametes only are formed, producing what he calls imperfect hybridization. No zygospores are produced; not even are gametes formed. The stimulus

from either strain is able to call forth the formation of progametes only in the other. Since he considers opposite strains to be zygotactic, he is thus able to find the sex of an unknown strain by growing it on a medium with a strain whose sex has previously been determined. Thus, if a strain whose sex is not known is grown on an agar plate with a plus strain, and if a sexual reaction is obtained, the new strain is considered to be minus. If no sexual reaction takes place, however, the strain is then contrasted with a minus strain; if sexual reaction is obtained, the new strain is considered plus; and if there is no sexual reaction with either the plus or minus strains, it is considered neutral. During his work BLAKESLEE found several strains of different species of Mucorineae which showed no reaction to either of his test species *Mucor V.* plus and minus. Those inactive strains he called neutrals, and believed them to be produced by the environment, because strains under artificial cultivation are sometimes found to lose their power of conjugation. HAGEM also found sexually inactive strains which he isolated from the soil. From 52 different strains of *Mucor* he found that 20 were minus, 3 were plus, and 29 were neutrals. If the Mucors are dioecious, as he and BLAKESLEE maintain, this seems to be a remarkably large percentage of neutrals to be found in a natural environment.

There are other conditions which may account for neutrals as well as the unfavorable environment. BURGEFF contrasted a plus and a minus strain of *Phycomyces nitens*, and from the sporangium of the germinating zygospore he obtained some spores which were plus, others which were minus, and a third kind which were neutral. The mycelium arising from the plus spores was also plus and produced plus spores. From the minus spores was obtained a minus mycelium, which in turn produced minus spores. On the other hand, from the neutral spores a mycelium was obtained which gave 3 kinds of spores, plus, minus, and neutral. In the neutral spores he believed the plus and minus nuclei to be of equal numbers. Thus the zygotactic stimulus of one kind of nuclei is counterbalanced by the nuclei of the opposite sex in the same hyphae.

In *Cunninghamella* there is exhibited a neutrality toward different strains which is unlike either of the neutral conditions

mentioned. The peculiarity shown by the different strains in their methods of conjugating with each other seems to indicate that the difference in sex in *Cunninghamella* is quantitative rather than qualitative. If one of the strains is taken as plus and all the others which conjugate with it as minus, there are strains which will conjugate with both plus and minus strains. On the other hand, there are, for example, strains (A) which will not show reaction with certain ones (B) but will in turn react with a third strain (C) which showed a sexual reaction with (B). The ability of one strain to show an individual selective power in conjugating with certain other strains is interpreted as evidence of a quantitative difference. Thus, if 2 strains are contrasted whose gametes are compatible a sexual reaction will take place. On the other hand, if 2 strains are contrasted whose gametes are incompatible no sexual reaction will take place.

Method

For two years I have been working with *Cunninghamella bertholletiae*. The cultures were obtained from decaying nuts of *Bertholletia excelsa*, together with cultures communicated to me by Dr. THAXTER, Dr. BLAKESLEE, and students in the Harvard Cryptogamic Laboratory. Authentic cultures of *C. bertholletiae* and *C. elegans* were obtained from Holland. The pure cultures used were obtained by transferring spores with a flamed needle directly from a single isolated head to a slant agar tube. Oatmeal agar was found to give the best results with these forms. The medium was made in the following manner: to every 1000 cc. of water was added 50 gm. of oatmeal. The mixture was steamed for 20 minutes, then strained through a triple thickness of cheesecloth, and 2 per cent of agar was then added. It was again autoclaved for 20 minutes at 15 lbs. pressure, after which it was tubed and sterilized.

The best results were obtained by placing the contrast series in battery jars, which were lined with moist filter paper and incubated at a temperature of 27° C. The contrast series were made by inoculating a Petri dish with 2 strains. It has been shown by BLAKESLEE that zygospores are formed more readily in moist

chambers kept at this temperature than under ordinary laboratory conditions. The tubes containing the pure cultures were treated in the same manner, but no zygospores appeared, showing that the cultures were pure and not a mixture of strains.

Cunninghamella has been regarded by BLAKESLEE as dioecious, and therefore no zygospores should appear unless both sexual strains are present. As both strains of *C. bertholletiae* had not yet been found, I contrasted all my cultures of this species with BLAKESLEE'S plus and minus strains of *C. echinulata*, hoping to secure imperfect hybridization, and thus, if possible, to determine the plus or minus nature of my strains. Many series of plates were made in which the cultures were contrasted with both strains of *C. echinulata*, but, although different media were used and the plates were subjected to different temperatures, I was unable to obtain imperfect hybridization in a single instance.

Both strains of BLAKESLEE'S *Mucor V.* were also used with a similar result. When *C. echinulata* plus and minus, as separated by BLAKESLEE, were contrasted with his *Mucor V.* plus and minus, imperfect hybridization took place. Moreover, when both the sexual strains of either of them, *Mucor V.* or *C. echinulata*, were themselves contrasted, the production of zygospores showed that the strains of these 2 test species had not lost their sexual vitality.

Experimental work

I have 34 different cultures of the Mucorineae which were contrasted with each other as indicated in table I. Cultures 1–26 inclusive are strains of *C. bertholletiae*, no. 21 being an authentic culture which was obtained from Holland. Cultures 27–31 are strains of *C. echinulata*, and of these 27 and 28 are *C. echinulata*, plus and minus as separated by BLAKESLEE. Cultures 32 and 33 are *Mucor V.* minus and plus as separated by BLAKESLEE, and 34 is an authentic culture of *C. elegans* which was also obtained from BLAKESLEE.

CONTRAST SERIES A

Having been unable to get any sexual reaction by contrasting the different strains of *C. bertholletiae* with *C. echinulata* plus and minus, I contrasted *C. elegans* no. 34 with all my other cultures.

TABLE I

SEXUALITY IN *Cunninghamella*

CONTRAST SERIES	C. BERTHOLLETIAE																									C. echinulata					Mucor V.		C. elegans
Cultures	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
Series A Culture 34			1					1				1	1								1			1	1	1						1		
Series B Culture 21	×	×	×				×		×	×			×	×		×	×			×														1
Series C Culture 9			×	×	×	×	×	×				×	×		×					×	×													
Series D Culture 14			×	×			×	×				×	×			×				×	×													
Series E Culture 7									×	×				×		×	×			×	×													
Series F Culture 3									×	×	×			×		×	×			×	×													1
Series G Culture 10			×				×	×				×	×					×	×		×	×	×											

As a result, imperfect hybridization took place between it and nos. 3, 8, 12, 13, 21, 24–26, and 32. Culture 25 is BLAKESLEE's *Mucor V.* minus, therefore the culture of *C. elegans* with which it reacted would be plus. It also reacted with culture 21, which is the authentic culture of *C. bertholletiae*. Since I was unable to get a sexual reaction with the different strains of *C. bertholletiae* when contrasted directly with *Mucor V.* plus and minus, I had to determine their sexual character by this indirect method. Since, therefore, *C. elegans* no. 34, because of its reaction with *Mucor V.* minus, would necessarily be plus, according to BLAKESLEE, *C. bertholletiae* no. 21 would be minus since it reacted with *C. elegans* no. 34. On the other hand, the cultures which did not react with no. 34 would be regarded as either plus or neutral.

CONTRAST SERIES B

Since I had thus determined that the sex of no. 21, the authentic culture of *C. bertholletiae*, was minus, I then contrasted it with all my cultures of this species, and normal zygospores were formed with nos. 1, 2, 3, 7, 9, 10, 13, 14, 16, 17, and 20, while imperfect hybridization took place again with no. 34, the authentic culture of *C. elegans*. All of these, therefore, on a basis of BLAKESLEE'S theory, should be regarded as certainly plus. In contrast series A, however, it was proved that cultures 3 and 13, like no. 21, were minus, but in the present series B they both formed normal zygospores when contrasted with no. 21. If we assume, therefore, that the species is dioecious, it is impossible on the theory of heterothallism to account for the production of normal zygospores in series B as a result of the interaction on no. 21 with mycelia which had been demonstrated to belong to the same sex.

CONTRAST SERIES C

As we have seen, cultures of *C. bertholletiae* no. 9 formed zygospores in series B with no. 21, which in series A was shown to be minus. *C. bertholletiae* no. 9, therefore, according to the theory of BLAKESLEE, should be regarded as plus. This plus strain no. 9 was then contrasted with all the other cultures of *C. bertholletiae*, and as a result formed zygospores with nos. 3–8, 12, 13, 15, 20, and 21. This presumably plus strain, therefore, formed zygo-

spores with nos. 3, 13, and 20, all 3 of which also formed zygospores in series B with no. 21, which in series A was shown to be minus. Since then, as determined by contrast with no. 21 in series B, nos. 3, 9, 13, and 20 are all plus, we have the anomaly of a plus strain no. 9 conjugating with 3 other plus strains, nos. 3, 13, and 20.

If we assume, as shown in series A and series B, that nos. 21 and 9 are minus and plus respectively, cultures 1, 2, 3, 7, 9, 10, 13, 14, 16, 17, and 20, since they react with no. 21, would be minus, and nos. 3–8, 12, 13, 15, 20, and 21, since they react with no. 9, would be plus. The other cultures, 22–33, which did not conjugate with either 21 or 9, must, according to BLAKESLEE, be considered as neutrals or as belonging to a different species. Cultures 3, 7, 13, and 20, on the other hand, must be hermaphroditic, or sexually bivalent, since they are able to conjugate with both plus and minus strains.

CONTRAST SERIES D

In this series culture 14, which was shown to be plus in series B, was contrasted with all the other cultures of *C. bertholletiae* and formed zygospores with nos. 3, 4, 7, 8, 12, 13, 16, 20, and 21. In series B cultures 9 and 14 were both shown to be plus. If these 2 cultures are plus, that is, if they are of the same sex, they should behave in the same manner when contrasted with all the other cultures. As a matter of fact, however, we see that no. 9 formed zygospores in series C with nos. 3–8, 12, 13, 15, 20, and 21, while in the present series culture 14 showed no sexual reaction with cultures 5, 6, and 15, but formed zygospores with 16. Since culture 9 does not form zygospores with the same cultures as no. 14, they cannot be regarded, therefore, as sexually identical.

CONTRAST SERIES E

Culture no. 7 was next used and formed zygospores with nos. 9, 10, 14, 16, 17, 20, and 21. This culture was shown to be plus in series B when contrasted with no. 21, and here again we notice a selective power, the reactions of this plus strain not conforming in all respects to the 2 plus strains employed in contrast series C and D.

CONTRAST SERIES F

In this series culture no. 3, which was shown to be plus in series B, was contrasted as in the previous series with all the remaining numbers and formed zygospores with nos. 9–11, 14, 16, 17, 20, and 21. Culture no. 11, which up to this time had failed to react with plus strains and had been considered neutral, formed normal zygospores when contrasted with no. 3. The conditions were the same as those of the previous experiments so far as it was possible to duplicate them. This series, as well as the others, was repeated several times, and in no instance did no. 11 form zygospores with 9, 14, or 21, the plus strains used in series B, C, and D.

CONTRAST SERIES G

Culture no. 10, which was determined to be plus in series B when contrasted on agar plates with the different strains, formed zygospores with nos. 3, 7, 8, 12, 13, 18, 19, 21–23. Cultures 18, 19, 22, and 23 had not formed zygospores with any of the other strains of *C. bertholletiae* previously tested, and therefore were considered as neutral. Since, however, in this series they formed zygospores with no. 10, they must be assumed to be minus. The question here arises, why if they are minus have they not conjugated with cultures nos. 3, 7, 9, and 14, which were all proved to be plus by the contrasts made in series B?

As had previously been stated, the cultures used in these 4 series were obtained from transfers made by touching single heads with a sterile needle and transferring directly, but in order to preclude the possibility of a mixture of strains, on January 3, 1917, single-spore cultures were made from cultures 3, 9, and 21, which were selected from the critical numbers used in the contrast series described. These cultures were made by the poured plate method. The germinating spores were located with the compound microscope, and were picked out by means of a fine needle and transferred to culture tubes. I obtained 3 single spore cultures of no. 3, 2 cultures of no. 9, and 4 cultures of no. 21. On January 27, 1917, the following contrast cultures were made with these single spore mycelia.

CONTRAST SERIES H

Each of the single-spore cultures of no. 3 were contrasted with each of the 2 pure cultures of no. 9, and in every case normal zygospores were produced, although both nos. 3 and 9 were shown in series B to be plus.

CONTRAST SERIES I

Each of the 3 sub-cultures of no. 3 were contrasted with each of the 4 cultures of no. 21, and every contrast plate produced normal zygospores, although the latter had been shown to be minus in series A.

CONTRAST SERIES J

The 2 sub-cultures of no. 9, which was shown to be plus in series B, were contrasted with each of the 4 sub-cultures of no. 21, and each contrast plate produced normal zygospores. It is demonstrated, therefore, beyond question that cultures 3, 9, and 21 form zygospores with both plus and minus strains, and therefore are sexually bivalent or hermaphroditic. Single-spore cultures were made from these 3 strains to eliminate the objection that the cultures had been mixed. Each of the cultures, obtained from a single spore, from either strain formed zygospores with the single-spore cultures of the other 2 strains, thereby confirming the results of the previous contrast series.

In the above contrast series, nos. 1 and 2 have always remained constantly plus, while nos. 4–6, 12, 15, 18, 19, 22–26 were always minus. Nos. 3, 7–11, 13, 14, 16, 17, 20, 21, and 34, however, have reacted with both the so-called plus and minus strains. Those numbers which showed no sexual reaction with any of the strains of *C. bertholletiae* are nos. 27–33, and therefore, according to the accepted terminology, are neutrals. We must remember, however, that it was pointed out before that cultures 27 and 28 are *C. echinulata* plus and minus as separated by BLAKESLEE. These 2 cultures always formed normal zygospores when contrasted with each other, and therefore are not neutrals. Cultures 32 and 33 are *Mucor V.* minus and plus as separated by BLAKESLEE; these cultures also formed normal zygospores when contrasted with

each other, and therefore are not considered as neutrals. Whenever cultures 25 and 32 were contrasted with culture 34, which is *C. elegans*, imperfect hybridization took place. It must also be remembered that cultures 25 and 32 are minus, as separated by BLAKESLEE. It is peculiar that the plus strains of *C. echinulata* and *Mucor V.* showed no sexual reaction with any of the cultures of *C. bertholletiae*.

Discussion

During the entire work, although careful search for them was made, no zygospores were obtained in the pure cultures, a fact which indicates that this species is not homothallic. Since the strains show a selective power in conjugating with other strains, however, it is not a heterothallic form as defined by BLAKESLEE.

Since it was impossible to get any of the strains of *C. bertholletiae* to react with *C. echinulata* or *Mucor V.* as separated by BLAKESLEE, I had to use the indirect method to determine the sex of the different strains. In contrast series A, no. 25, which is *Mucor V.* minus, showed a sexual reaction with no. 34, which is an authentic culture of *C. elegans*. All the cultures which did not react with no. 34 must therefore be considered either of the same sex, which is minus or neutral.

In contrast series B culture no. 21 formed normal zygospores with cultures 3 and 13, which themselves were proved in contrast series A to be of the same sex as 21.

On the assumption that the species is dioecious, it is impossible to account for the production of normal zygospores as a result of the interaction of mycelia of the same "sex." The situation is further complicated by the behavior of some of the so-called "neutral strains."

On the basis of contrast series B, nos. 1, 2, 3, 7, 9, 10, 13, 14, 16, 17, and 20 are plus. On the other hand, in contrast series A nos. 3, 8, 12, 13, 21, 24–26, and 32 were shown to be minus. According to BLAKESLEE'S views, nos. 4–6, 11, 15, 18, 19, 22, 23, 27, 28, 30, 31, and 33 must be neutral, for they show no sexual reaction with 2 strains which were shown to be plus and minus respectively.

The "neutrals" 4–6 and 15, however, formed zygospores when contrasted with culture no. 9 in series C. Since 9 was proved in series B to be plus, nos. 4–6 and 15 would therefore be considered as minus.

The neutral no. 11 formed normal zygospores when contrasted with 3 in series F. We have shown, however, that 3 is plus in series B and minus in series A; therefore no. 3 would have to be considered hermaphroditic.

Nos. 18, 19, 22, and 23, which were shown to be neutrals, formed normal zygospores when contrasted with no. 10 in series G. No. 10 was shown to be plus in series B; therefore nos. 18, 19, 22, and 23 must be considered as minus. If these numbers are considered as minus, the question arises, why did they not conjugate with nos. 34, 21, 9, 7, and 3, which were all shown to be plus?

The evidence that nos. 4–6, 11, 15, 18, 19, 22, 23, 27, 28, 30, 31, and 33 are "neutral" does not seem to fit the accepted conception of this term, for they are able to form normal zygospores when contrasted with the strains whose gametes are compatible.

If plus and minus strains of the *Mucor V.* are female and male respectively, they should have shown a sexual reaction with different strains of *C. bertholletiae* which were capable of forming zygospores when contrasted with each other. This neutrality cannot be explained on the grounds of loss of vitality; neither do I believe this neutrality can be explained by BURGEFF'S hypothesis, that there are the same number of plus and minus nuclei in a given hypha, the plus nuclei annulling the zygotactic influence of the minus. It has been shown that a strain which is neutral to 2 strains, which themselves are plus and minus respectively, has the power to conjugate with a third strain if their gametes are compatible. The writer is unable at the present time to give any satisfactory explanation of this pseudo-heterothallic condition in *Cunninghamella bertholletiae*, but it is evident that we still have much to learn as to the sexual conditions in the Mucorineae, especially in relation to so-called neutral strains.

Summary

1. In *Cunninghamella* there does not exist sexual dimorphism.

2. *C. echinulata* plus and minus, or *Mucor V.* plus and minus as separated by BLAKESLEE, are unable to form progametes or gametes when contrasted with any one of 26 cultures of *C. bertholletiae*.

3. Many of these cultures of *C. bertholletiae* were able to form zygospores when contrasted with certain other cultures of this same species.

4. There exists a selective power in some strains to form zygospores with certain other strains. This condition of pseudo-heterothallism cannot be explained at present.

5. There exists a condition in some strains which might be called hermaphroditism.

6. In none of the hermaphroditic strains did branches of the same hyphae conjugate.

7. Zygospores were produced only when 2 strains were contracted whose gametes were compatible.

I wish to express my gratitude to Professor THAXTER, under whose direction the work was undertaken, also to Dr. BLAKESLEE and Mr. A. R. BUTLER for various cultures used in this study.

HARVARD UNIVERSITY
CAMBRIDGE, MASS.